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Biosynthesis, transport, and modification of lipid A[1,2]

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Abstract: Lipopolysaccharide (LPS) is the major surface molecule of Gram-negative bacteria and consists of three distinct structural domains: O-antigen, core, and lipid A. The lipid A (endotoxin) domain of LPS is a unique, glucosamine-based phospholipid that serves as the hydrophobic anchor of LPS and is the bioactive component of the molecule that is associated with Gram-negative septic shock. The structural genes encoding the enzymes required for the biosynthesis of *Escherchia coli* lipid A have been identified and characterized. Lipid A is often viewed as a constitutively synthesized structural molecule. However, determination of the exact chemical structures of lipid A from diverse Gram-negative bacteria shows that the molecule can be further modified in response to environmental stimuli. These modifications have been implicated in virulence of pathogenic Gram-negative bacteria and represent one of the molecular mechanisms of microbial surface remodeling used by bacteria to help evade the innate immune response. The intent of this review is to discuss the enzymatic machinery involved in the biosynthesis of lipid A, transport of the molecule, and finally, those enzymes involved in the modification of its structure in response to environmental stimuli.

Key words: lipopolysaccharides, lipid A, endotoxin, outer membrane, MsbA.

Résumé : Le lipopolysaccharide (LPS) est la principale molécule à la surface des bactéries à Gram négatif et comporte trois domaines structuraux différents : l'antigène O, le cœur et le lipide A. Le domaine du lipide A (endotoxine) du LPS est un phospholipide particulier, ayant la glucosamine comme base, qui est l'ancre hydrophobe du LPS et le constituant actif de la molécule associée au choc septique des bactéries à Gram négatif. Les gènes structuraux codant les enzymes essentielles à la biosynthèse du lipide A de *Escherichia coli* ont été identifiés et caractérisés. Le lipide A est souvent considéré comme étant une molécule structurale synthétisée de façon constitutive. Cependant, la détermination de la structure chimique exacte du lipide A de diverses bactéries à Gram négatif montre que la molécule peut être modifiée à la suite de stimulus environnementaux. Ces modifications interviendraient dans la virulence des bactéries pathogènes à Gram négatif et elles représentent un des mécanismes moléculaires de remodelage de la surface microbienne utilizé par les bactéries pour tenter de déjouer la réponse immune innée. Cette revue a pour but de discuter du mécanisme enzymatique intervenant dans la biosynthèse et le transport du lipide A, ainsi que des enzymes intervenant dans la modification de sa structure à la suite de stimulus environnementaux.

Mots clés : lipopolysaccharides, lipide A, endotoxine, membrane externe, MsbA.

[Traduit par la Rédaction]

Introduction

It has been over 20 years since Masahiro Nishijima and Christian R.H. Raetz reported the accumulation of a membrane-associate glycolipid, lipid X, from an *Escherichia coli* mutant deficient in phosphatidylglycerol (Nishijima and Raetz 1979, 1981). The discovery of this unique lipid eventually led to the elucidation of the lipid A biosynthetic pathway, which includes nine biosynthetic enzymes. Significant advances have also been made in understanding the transport of lipid A to the bacterial outer surface where it resides as the hydrophobic anchor of lipopolysaccharide (LPS).

Unlike Gram-positive organisms, Gram-negative bacteria are further guarded from their environment by an asymmetric outer membrane that encapsulates their peptidoglycan (Fig. 1). The inner leaflet of the outer membrane is composed of phospholipids, primarily phosphatidylethanolamine, whereas the outer leaflet is composed mainly of a unique immunogenic glycolipid called LPS. LPS is the major surface molecule of Gram-negative bacteria and consists of three distinct structural domains: O-antigen, core, and lipid A (Fig. 1). Lipid A (endotoxin) functions as the hydro-

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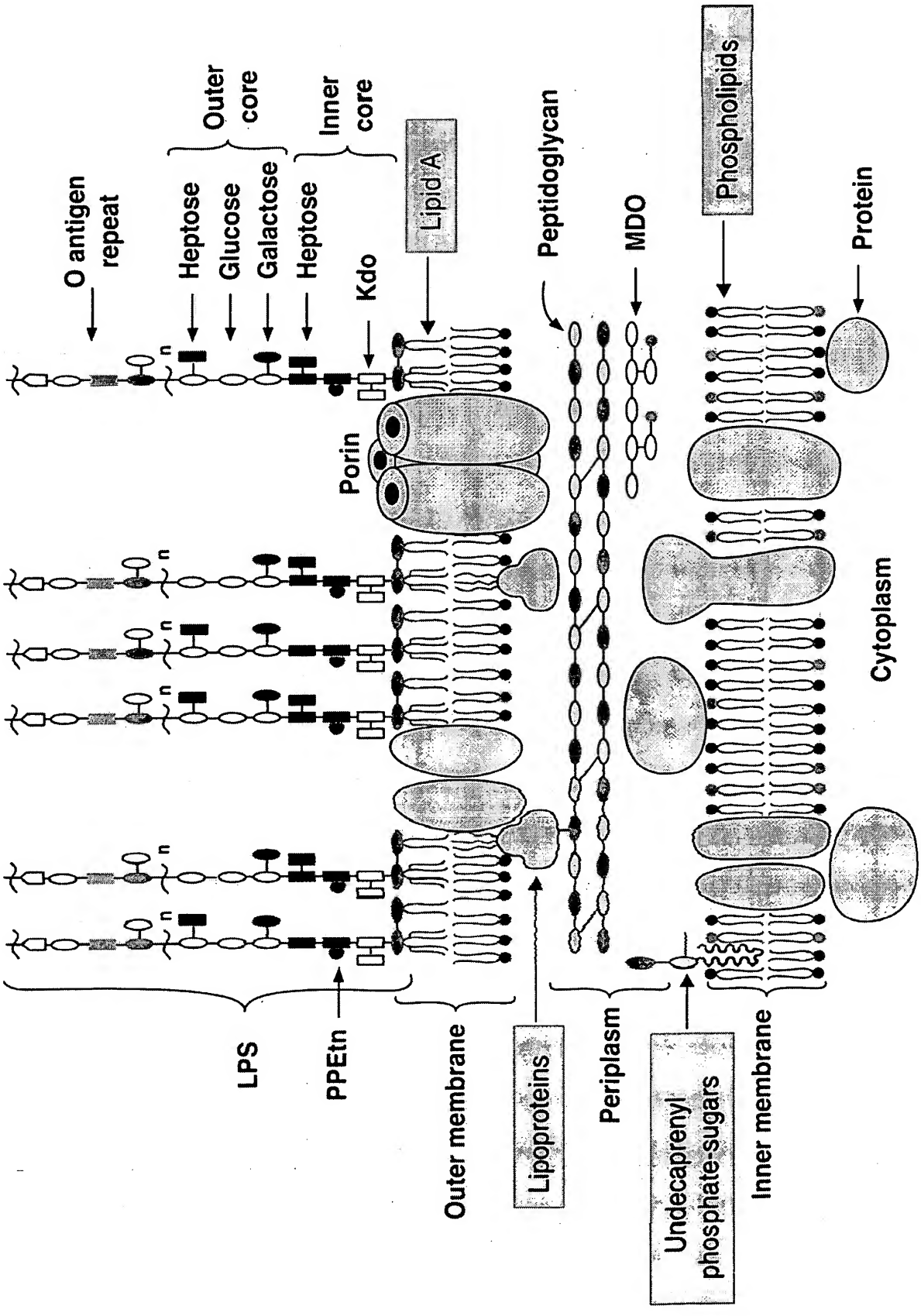


Fig. 1. Schematic of the Gram-negative cell envelope typified by *Escherichia coli*. The peptidoglycan layer of Gram-negative bacteria is enclosed by an asymmetric lipid bilayer, interspersed with proteins. The inner leaflet is composed of glycerophospholipids whereas the outer leaflet of the outer membrane is composed almost exclusively of lipopolysaccharide (LPS). Lipid A serves as the hydrophobic anchor of LPS. MDO are membrane-derived oligosaccharides. Figure courtesy of C.R.H. Raetz. (Reprinted, with permission, from the *Annual Review of Biochemistry*, Volume 71 ©2002 by Annual Reviews www.annualreviews.org.) ppEtn, 2-aminoethyl pyrophosphate.

phobic anchor of LPS and is the bioactive component of the molecule that is associated with Gram-negative septic shock (Raetz 1996).

The lipid A of *E. coli* and *Salmonella typhimurium* is a β-1′-6-linked disaccharide of glucosamine, phosphorylated at positions 1 and 4′ and acylated at the 2, 3, 2′, and 3′ positions of the disaccharide with *R*-3-hydroxymyristate. The hydroxyl groups of the 2′- and 3′-linked fatty acyl chains are further esterified with laurate (C12) and myristate (C14), respectively, and the molecule is glycosylated at the 6′ position with two 3-deoxy-D-*manno*-octulosonic acid (Kdo) moieties (Fig. 2). In wild-type strains, attached to the Kdo moiety are the core and O-antigen sugars consisting of polysaccharide chains (Raetz 1996) (Fig. 1). Typical *E. coli* K12 laboratory strains do not contain the O-antigen region of LPS (Schnaitman and Klena 1993; Whitfield 1995). Lipid A is required to maintain the integrity of the outer membrane barrier (Galloway and Raetz 1990; Vaara 1993), and the minimal structure required for *E. coli* cell growth under normal laboratory conditions is the Kdo-modified hexa-acylated lipid A (Raetz and Whitfield 2002; Raetz 1996). However, the polysaccharide core and O-antigen regions of LPS are important for antibiotic resistance, evasion of the complement system, and various environmental stresses (Nikaido 1994). To date, the nine constitutive enzymes catalyzing the reactions involved in the biosynthetic pathway of Kdo_2 – lipid A have been discovered (Babinski et al. 2002*a*, 2002*b*; Raetz and Whitfield 2002). The biosynthetic pathway of *E. coli* lipid A is shown in Fig. 2.

During animal infections, lipid A interacts with a variety of cell- and serum-binding proteins, leading to the activation of the innate immune system. Initially, the presence of lipid A is sensed by LPS-binding protein, a serum protein that delivers lipid A to CD14 found on the surfaces of animal cells (Tobias et al. 1986; Ulevitch and Tobias 1999). Lipid A or the lipid A – CD14 complex interacts with the pattern-recognition receptor Toll-like receptor 4 that is responsible for signal transduction (Aderem and Ulevitch 2000; Hoshino et al. 1999). The host response to lipid A includes the production of cytokines and clotting factors and the synthesis and secretion of cationic antimicrobial peptides (CAMPs) and additional stimulatory molecules (Aderem and Ulevitch 2000; Esmon 2000; Medzhitov and Janeway 1998, 2000). In limited infections, the response to lipid A helps to clear the invading microbe. However, in overwhelming sepsis, high levels of circulating cytokines and procoagulant activity may damage the microvasculature and precipitate the syndrome of septic shock, leading to multiple organ failure and death (Parillo 1993; van Deuren et al. 2000). Approximately half of the documented infections in patients with sepsis are Gram-negative bacterial infections (Young 1995). Since lipid A is required for bacterial growth, inhibitory agents targeting its synthesis should be bactericidal for most Gram-negative bacteria. Since these agents would decrease lipid A production, their effects would be to inhibit bacterial growth, increase the permeability of the outer membrane to antibiotics, and decrease the host immune response associated with septic shock by endotoxin. Inhibition of any of the first seven steps of lipid A biosynthesis in *E. coli* results in a loss of cell viability (Raetz and Whitfield 2002; Raetz 1996).

The lipid A biosynthetic pathway

The enzymology and molecular genetics of lipid A biosynthesis have been primarily carried out by Raetz and coworkers using *E. coli* as the representative organism. The lipid A pathway, also known as the Raetz pathway (Fig. 2), consists of nine enzymatic steps and is conserved among Gram-negative bacteria. The first step, catalyzed by LpxA, is the acylation of the sugar nucleotide UDP-*N*-acetylglucosamine (UDP-GlcNAc) (Coleman and Raetz 1988; Galloway and Raetz 1990). Fatty acylation of sugar nucleotides had not been reported previously in *E. coli* or other organisms. The UDP-GlcNAc substrate sits at a unique branch point in the metabolism of Gram-negative bacteria, since the compound is a precursor to both the peptidoglycan framework and the synthesis of lipid A and, therefore, LPS.

Escherichia coli LpxA is highly selective for β-hydroxymyristate and uses only acyl carrier protein as the obligate acyl donor (Williamson et al. 1991; Wyckoff et al. 1998). Substrate specificity of LpxA is variable in different bacteria and contributes to the structural differences seen in lipid A from various organisms (Dotson et al. 1998; Odegaard et al. 1997; Sweet et al. 2001, 2002; Wyckoff et al. 1998). For example, the LpxA of *Chlamydia trachomatis* utilizes myristoyl-ACP rather than hydroxymyristoyl-ACP (Sweet et al. 2001). The crystal structure of *E. coli* LpxA has been solved and shows a homotrimeric structure with a unique protein fold characterized by a left-handed helix of short parallel β-sheets (Pfitzner et al. 1995; Raetz and Roderick 1995). Although the enzymatic activity of LpxA from various organisms has been investigated, the structural basis for the acyl-chain selectivity of the enzyme still merits further investigation.

The first committed step of lipid A biosynthesis is catalyzed by the zinc metalloenzyme LpxC, the UDP-monoacyl-GlcNAc deacetyalse. The enzyme catalyzes the irreversible hydrolysis of the amide linkage at position 2 of the glucosamine sugar by removing an acetyl group (Anderson et al. 1993; Kelly et al. 1993; Young et al. 1995). LpxC has been purified from *E. coli*, the opportunistic pathogen *Pseudomonas aeruginosa*, and the thermophilic Gram-negative bacterium *Aquifex aeolicus* (Hyland et al. 1997; Jackman et al. 1999, 2001). The *lpxC* gene is highly conserved in diverse Gram-negative bacteria and has no homology to mammalian deacetylases or amidases, making it an attractive target for new antibacterial compounds. Indeed, compounds containing hydroxyamate or phosphonate zinc-binding motifs have been explored as potent LpxC inhibitors (Jackman et al. 2000; Kline et al. 2002; Li et al. 2003; Onishi et al. 1996; Pirrung et al. 2002, 2003). Within the last year, both the crystal structure and the nuclear magnetic resonance structure of LpxC from *A. aeolicus* were solved. The 32-kDa enzyme is characterized by a novel α/β fold and a unique zinc-binding motif, strengthening the observation that it lacks both sequence and structural homology to other metalloamidases (Coggins et al. 2003; Whittington et al. 2003). Also of interest is the so-called "hydrophobic passage" that is proposed to bind to the fatty acyl chain of the UDP-3-monoacyl-GlcNAc substrate (Coggins et al. 2003). Elucidation of the LpxC structure may aid in the development of LpxC inhibitors active against a broad spectrum of Gram-negative bacteria.

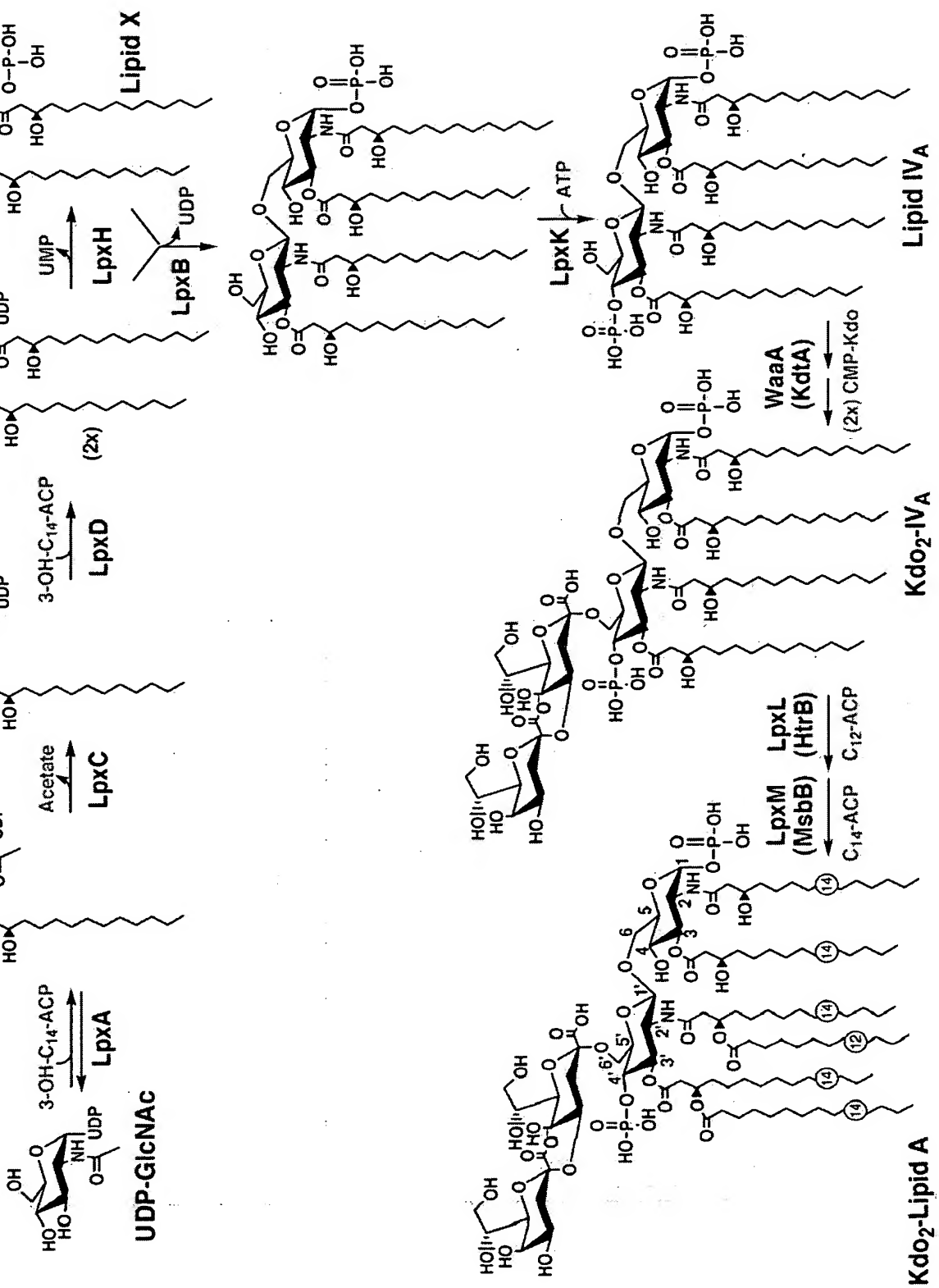


Fig. 2. Constitutive lipid A (endotoxin) pathway, the Raetz pathway. The structures shown are for the biosynthesis of Kdo_2 – lipid A in *Escherichia coli* K-12 and *Salmonella* typhimurium. All of the major intermediates are named (blue) and a single enzyme (red) catalyzes each reaction. The lengths of the fatty acyl chains are indicated by enclosed circles. Acyl-ACP (acyl carrier protein) serves as the obligate acyl donor for the various acyltransferases.

LpxC-catalyzed removal of the acetyl group from position 2 allows for the addition of a second fatty acyl chain in an amide linkage to form UDP-2,3-diacylglucosamine. This second acylation is catalyzed by LpxD, a 36-kDa protein that shows significant sequence homology to LpxA. The *lpxD* gene, located at minute 4 of the *E. coli* chromosome, is part of the same complex operon as *lpxA* and *lpxB* (Kelly et al. 1993). Originally, LpxD (also known as FirA) was thought to interact with the β-subunit of RNA polymerase as part of the machinery involved in transcription. Based on homology to *lpxA*, Kelly et al. (1993) investigated the role of *lpxD*/*firA* in lipid A biosynthesis and demonstrated its activity as the *N*-acyltransferase and confirmed that a mutant *lpxD* allele confers a defect in lipid A synthesis.

The fourth step of Kdo_2 – lipid A biosynthesis is catalyzed by the enzyme LpxH. LpxH cleaves the pyrophosphate bond of the UDP-2,3-diacylglucosamine intermediate, the precursor of the nonreducing sugar of lipid A, yielding UMP and 2,3-diacylglucosamine 1-phosphate (lipid X) (Babinski et al. 2002*a*, 2002*b*). The *lpxH* gene was the last of the structural genes of the nine enzymatic steps to be discovered. In contrast with the key structural genes in the lipid A pathway, clear orthologs of *lpxH* are only found in approximately 50% of the Gram-negative organisms sequenced to date. However, more distant orthologs designated as *lpxH2* can be found in other Gram-negative bacteria (Babinski et al. 2002*b*). Clearly, bacteria lacking LpxH contain an unidentified UDP-2,3-diacylglucoasamine-specific pyrophosphatase.

Formation of the characteristic β-1′-6 glycosidic linkage present in all lipid A molecules is catalyzed by LpxB, the dissacharide synthase. The dimeric enzyme condenses one molecule of UDP-2,3-diacylglucosamine with one molecule of 2,3-diacylglucosamine 1-phosphate, with the latter serving as the acceptor molecule. The structural gene for the enzyme was first discovered during attempts to generate phosphatidylglycerol-deficient mutants in *E. coli* already harboring a mutation in the structural gene for phosphatidylglycerol synthetase, *pgsA*. The reason that *E. coli* strains harboring an *lpxB* (*pgsB*) mutation lack phosphatidylglycerol still remains unclear. Babinski and co-workers constructed an *lpxH* insertion mutant that was nonviable and accumulated UDP-2,3-diacylglucosamine, thus supporting the observation that LpxB condenses one molecule of UDP-2,3-diacylglucosamine and one molecule of 2,3-diacylglucosamine 1-phosphate (Fig. 2). Up until the formation of the lipid A dissacharide backbone, the subcellular localization of the lipid A biosynthetic enzymes is cytosolic (Anderson et al. 1993; Kelly et al. 1993; Young et al. 1995). LpxH and LpxB activities actually partition between cytosolic and membrane fractions upon ultracentrifugation but do not require detergent for activity (Babinski et al. 2002*a*, 2002*b*; Radika and Raetz 1988; Ray et al. 1984). The latter steps of the pathway catalyzed by LpxK, WaaA (KdtA), LpxL, and LpxM all require nonionic detergents for activity and are membrane bound (Belunis et al. 1992; Clementz et al. 1996, 1997; Ray and Raetz 1987).

After the formation of the dissacharide backbone, LpxK, a specific kinase, catalyzes the ATP-dependent phosphorylation of the 4′-hydroxyl group of the tetraacyldisacharide 1-phosphate intermediate, which results in the formation of a key lipid A precursor, lipid IV_A. This enzyme requires Mg^{2+} for activity, is stimulated by cardiolipin, and is specific for glucosamine disaccharides (Garrett et al. 1997, 1998; Ray and Raetz 1987). The LpxK amino acid sequence shows no obvious homology to other kinases and displays two potential membrane-spanning domains. LpxK-catalyzed phosphorylation is thought not to occur prior to Kdo addition, since lipid IV_A accumulates in mutants unable to incorporate Kdo into lipid A (Clementz and Raetz 1991; Goldman et al. 1988*a*; Raetz et al. 1985; Rick et al. 1977; Strain et al. 1985). Futhermore, 4′-dephosphorylated Kdo_2 – lipid IV_A is not a substrate for membranes from cells overexpressing LpxK (Brozek et al. 1989; Garrett et al. 1997). Cloning of LpxK allowed for the production of ^{32}P-labeled lipid A precursor substrates with high specific activity, thus facilitating the discovery and characterization of enzymes involved in the latter stages of lipid A biosynthesis and its modification (discussed later).

In *E. coli* the seventh step of Kdo_2 – lipid A biosynthesis is the transfer of the Kdo sugar to the 6′ position of the disacharide backbone. The reaction, catalyzed by WaaA (formerly KdtA) (Clementz and Raetz 1991), uses CMP-Kdo as the nucleotide sugar donor (Raetz 1990; Raetz and Whitfield 2002; Raetz 1996). WaaA of *E. coli* is a bifunctional glycosyltransferase that catalyzes the addition of two Kdo residues having distinct glycosidic linkages (Belunis and Raetz 1992; Clementz and Raetz 1991). The addition of Kdo to the 6′-OH of lipid IV_A serves as the first step of core biosynthesis in Gram-negative bacteria with the 5-OH of the inner Kdo as the point of attachment of additional core sugars (Raetz and Whitfield 2002; Raetz 1996). WaaA has been identified and characterized in several organisms, including *E. coli* (Belunis et al. 1995; Clementz and Raetz 1991), *C. trachomatis* (Belunis et al. 1992), and *Haemophilus influenzae* (Brabetz et al. 2000*b*; White et al. 1997). In *C. trachomatis*, the enzyme transfers three Kdo residues to the lipid A backbone, which forms a genus-specific LPS epitope (Belunis et al. 1992). Up to four Kdo residues can be found in an *E. coli waaA* mutant expressing the *Chlamydophila psittaci* homologue (Brabetz et al. 2000*a*). *Haemophilus influenzae* WaaA catalyzes the addition of one Kdo sugar, which is then phosphorylated by KdkA (Kdo kinase A) at the 4-OH position typically occupied by the second Kdo in most Gram-negative bacteria (Brabetz et al. 2000*b*; White et al. 1997, 1999). The enzymatic functionality of the various Kdo transferases is quite remarkable, and comparison of their secondary structures gives no clues as to the number of glycosidic linkages that they can create. Interestingly, investigation of the LPS of certain *E. coli* strains (with K-12 and R2 core types) and *Salmonella enterica* serovars shows the nonstoichiometric addition of a third Kdo sugar to the outer Kdo residue (Heinrichs et al. 1998; Kaniuk et al. 2002; Muller-Loennies et al. 2003). Frirdich et al. (2003) have demonstrated that *waaZ*, a gene product found in the *waa* locus, is required for the addition of the third Kdo. It is still unclear whether WaaZ serves as a secondary Kdo transferase associated with specific core types or plays a role in the regulation of an unidentified Kdo transferase.

In *E. coli*, the synthesis of lipid A is completed by the addition of two fatty acyl chains to the distal glucosamine at the 2′ and 3′ positions, thus forming an asymmetric molecule

(Fig. 3). The so-called "late" acyltransferases, LpxL (HtrB) and LpxM (MsbB), catalyze the transfer of laurate (C12) and myristate (C14), respectively (Clementz et al. 1996, 1997; Raetz and Whitfield 2002). Although the enzymes use acyl-ACPs as the obligate substrate, they show absolutely no homology to earlier acyltransferases in the lipid A pathway (Fig. 2) (Clementz et al. 1996, 1997). LpxM and LpxL do, however, show significant similarity to each other. The late acyltransferases of *E. coli* absolutely require the presence of the Kdo disaccharide for activity, and the addition of the lauroyl residue to Kdo_2 – lipid IV_A precursor precedes the incorporation of myristoyl residues (Clementz et al. 1996, 1997).

However, incorporation of laurate into lipid A does not require prior attachment of Kdo in all Gram-negative bacteria, as is the case in *P. aeruginosa* (Goldman et al. 1988*b*; Mohan and Raetz 1994) and in *Neisseria meningitidis* (Tzeng et al. 2002). The late acyltransferases of these organisms do not require the Kdo disaccharide and transfer one laurate chain to each glucosamine unit at the 2 and 2′ positions to form a more symmetrical lipid A (Fig 3). Inhibition of Kdo addition in *P. aeruginosa* using inhibitors of Kdo synthesis leads to the accumulation of hexa-acylated lipid A and results in cell death (Goldman et al. 1988*b*). Inactivation of the meningococcal *waaA* gene also results in organisms producing hexa-acylated lipid A, but the mutation is viable (Tzeng et al. 2002). Interestingly, unlike most Gram-negative human pathogens, *N. meningitidis* does not require lipid A for growth (Steeghs et al. 1998; van der Ley and Steeghs 2003). *Neisseria meningitidis lpxA* insertion mutants are viable and possess an outer membrane containing integral outer membrane proteins (Steeghs et al. 2001; van der Ley and Steeghs 2003). However, the LPS-deficient mutant has a decline in growth rate, and presence of the capsular polysaccharide is essential for cell viability, suggesting that the polysaccharide may substitute for lipid A (Steeghs et al. 1998; van der Ley and Steeghs 2003).

The acyloxyacyl groups of lipid A are important for the endotoxic properties of the molecule (Alexander and Rietschel 2001; Golenbock et al. 1991; Loppnow et al. 1989). For this reason, late acyltransferase mutants of various pathogens have been constructed in a number of organisms for possible use in vaccine development. In *E. coli*, inactivation of *lpxM* results in the synthesis of pent-acylated lipid A lacking myristate residues, but the mutation is not lethal (Karow et al. 1992). Mutation of *lpxM* in a virulent *E. coli* strain (Somerville et al. 1999) or in *Salmonella* significantly reduces the virulence of the bacterium in animal models (Khan et al. 1998; Low et al. 1999). LpxL and LpxM of *H. influenzae* are both myristoyl transferases resulting in the typical hexa-acylated lipid A. Inactivation of *H. influenzae lpxL* (*htrB*) results in the production of a lipid A that is 90% tetra-acylated as well as a decrease in modification of the core structure with phosphoethanolamine (Lee et al. 1995). The *H. influenzae lpxL* mutants are attenuated in infection studies in infant rat models (Nichols et al. 1997) and have decreased ability to colonize human airway xenografts (Swords et al. 2002). Although it appears that encapsulated Neisseriaceae do not require lipid A for growth (Steeghs et al. 1998), loss of the acyloxyacyl moieties from their lipid A is critical for the induction of full inflammatory responses (Dixon et al. 2001; Ellis et al. 2001) and makes the diplococci more susceptible to innate intracellular killing mechanisms (Post et al. 2002). Late acyltransferase mutants of various pathogens have been pursued for their use in vaccine development.

Transport of lipid A

Following the late acylation events of lipid A, the core sugars are attached to the inner Kdo sugar. The enzymatic machinery responsible for the assembly of the core – lipid A structure is located in the cytoplasm or on the inner surface of the inner membrane (Raetz and Whitfield 2002; Raetz 1996). Eventually, mature LPS comprising the lipid A anchor, the core oligosaccharide, and the O-antigen reach their destination to the outer surface of the outer membrane. The outer membrane is an asymmetric bilayer with phospholipids on its inner surface and LPS on the outside surface. The mechanisms by which components of the outer membrane reach their destination are largely unknown. However, in the last several years, significant steps that help to define the process have been taken.

Insights into the transport of lipid A and bacterial phospholipids across a membrane barrier were first revealed in the generation of *lpxL* (*htrB*) mutants. At 42 °C, *E. coli lpxL* mutants accumulate LPSs having tetra-acylated lipid A species in the inner membrane, leading to a loss of cell viability (Karow et al. 1991). The *E. coli msbA* gene was found to serve as a second site suppressor of the *lpxL* mutation at 42 °C, restoring growth even with a tetra-acylated lipid A anchor (Polissi and Georgopoulos 1996; Zhou et al. 1998). MsbA is an essential ABC transporter sharing homology with the multidrug resistance proteins of eukaryotes (Doerrler and Raetz 2002; Doerrler et al. 2001; Zhou et al. 1998). ABC transporters are composed minimally of two transmembrane domains and two nucleotide binding domains. *Escherichia coli* MsbA is a homodimer containing six transmembrane domains (Chang 2003; Chang and Roth 2001). A novel *E. coli* temperature-sensitive mutant with a single point mutation in the fifth transmembrane region of MsbA results in the rapid inhibition of phospholipid and LPS export to the outer membrane (Doerrler et al. 2001). Studies in vitro have shown that *E. coli* MsbA purified to near homogeneity is an ATPase that is specifically stimulated by hexa-acylated lipid A and Kdo_2 – lipid A. The lipid A precursors lipid IV_A (tetra-acylated) and lipid X (2,3-diacylglucosamine 1-phosphate) did not stimulate ATPase activity (Doerrler and Raetz 2002). However, Kdo_2 – lipid IV_A must serve as a substrate in vivo, since overexpression of MsbA restores growth to *E. coli lpxL* mutants at 42 °C.

Escherichia coli MsbA was the first ABC transporter to be crystallized and analyzed by X-ray crystallography by Chang and Roth (2001), resulting in a protein structure at a resolution of 4.5 Å. More recently, the X-ray structure of MsbA from *Vibrio cholera* was determined to 3.8 Å (Chang 2003). The two flippases share a structurally conserved core of transmembrane α-helices but differ in the relative orientations of their nucleotide-binding domains (Chang 2003; Chang and Roth 2001). The two halves of the transmembrane domain form the proposed lipid A and (or) phospholipid "flipping chamber". The chamber is lined with basic

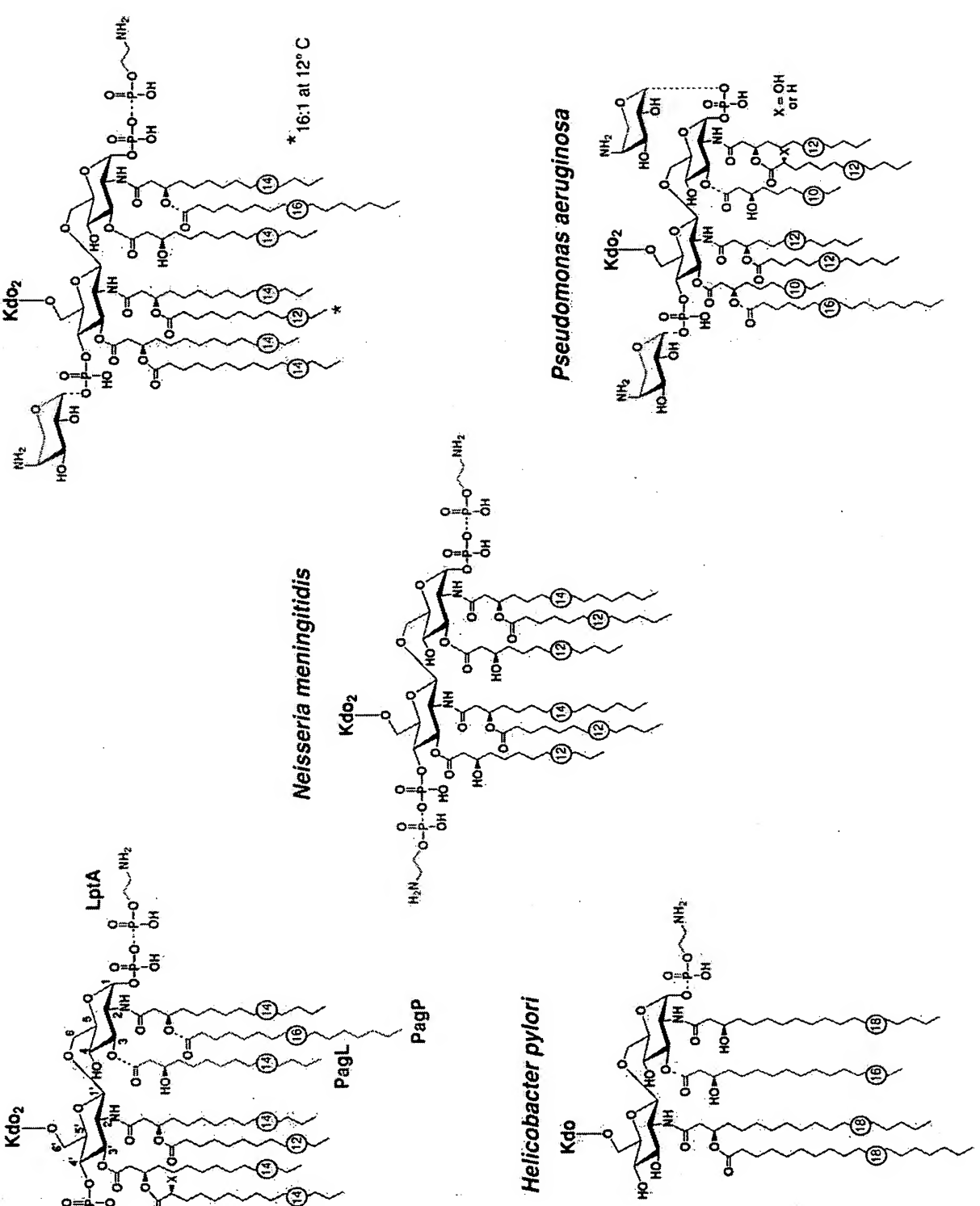


Fig. 3. Lipid A structures of various pathogenic Gram-negative bacteria. Partial covalent modifications are indicated with dashed bonds and acyl chain lengths are indicated by enclosed circles. In *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*, modifications to the lipid A structure have been shown to be regulated by two-component regulatory systems (Ernst et al. 1999; Gunn et al. 1998; Guo et al. 1997, 1998). Structural proteins required for the modification of lipid A are indicated. No enzymatic data have been presented for the lipid A hydroxylase LpxO (Gibbons et al. 2000). A phosphoethanolamine transferase activity has been identified in membranes of polymyxin-resistant mutants of *E. coli* (M.S. Trent, M. Reynolds, and C.R.H. Raetz, unpublished data).

residues and is open to the intracellular side of the membrane. Recruitment of the lipid A substrate into the flipping chamber is proposed to induce nucleotide binding followed by dimerization of the two nucleotide-binding domains where it flips spontaneously. Following ATP hydrolysis, the so-called "flipping chamber" opens to release the lipid substrate to the periplasmic side of the inner membrane (Chang 2003; Chang and Roth 2001). Reuter et al. (2003) recently showed that the ABC multidrug transporter LmrA of *Lactococcus lactis* serves as a functional homologue of *E. coli* MsbA. Interestingly, the ATPase activity of LmrA was lipid A stimulated (Reuter et al. 2003). Taken together, these data suggest that LmrA is capable of transporting lipid A in *E. coli*, although *L. lactis* is a Gram-positive bacterium containing no LPS. It would be worthwhile to investigate the lipid profiles of the *E. coli msbA* mutant complemented with *lmrA* to determine if normal LPS transport takes place. Elucidation of the protein structure of MsbA and insights into its function have been remarkable developments in the field of LPS and ABC transporters.

Regulated covalent modifications of LPS

Addition, removal, and modification of lipid A fatty acyl chains

Nine enzymes are required for biosynthesis of Kdo_2 – lipid A, the minimal LPS required for *E. coli* growth under normal laboratory conditions. With few exceptions, single copies of the lipid A biosynthetic genes are present in all Gram-negative bacteria. Although the overall lipid A structure is relatively conserved among various bacteria, its structure is modified in response to the local environment, which, in turn, results in changes in the outer surface of the bacterium. In general, modifications of the lipid A structure include the removal or decoration of the lipid A phosphates and fatty acyl chains.

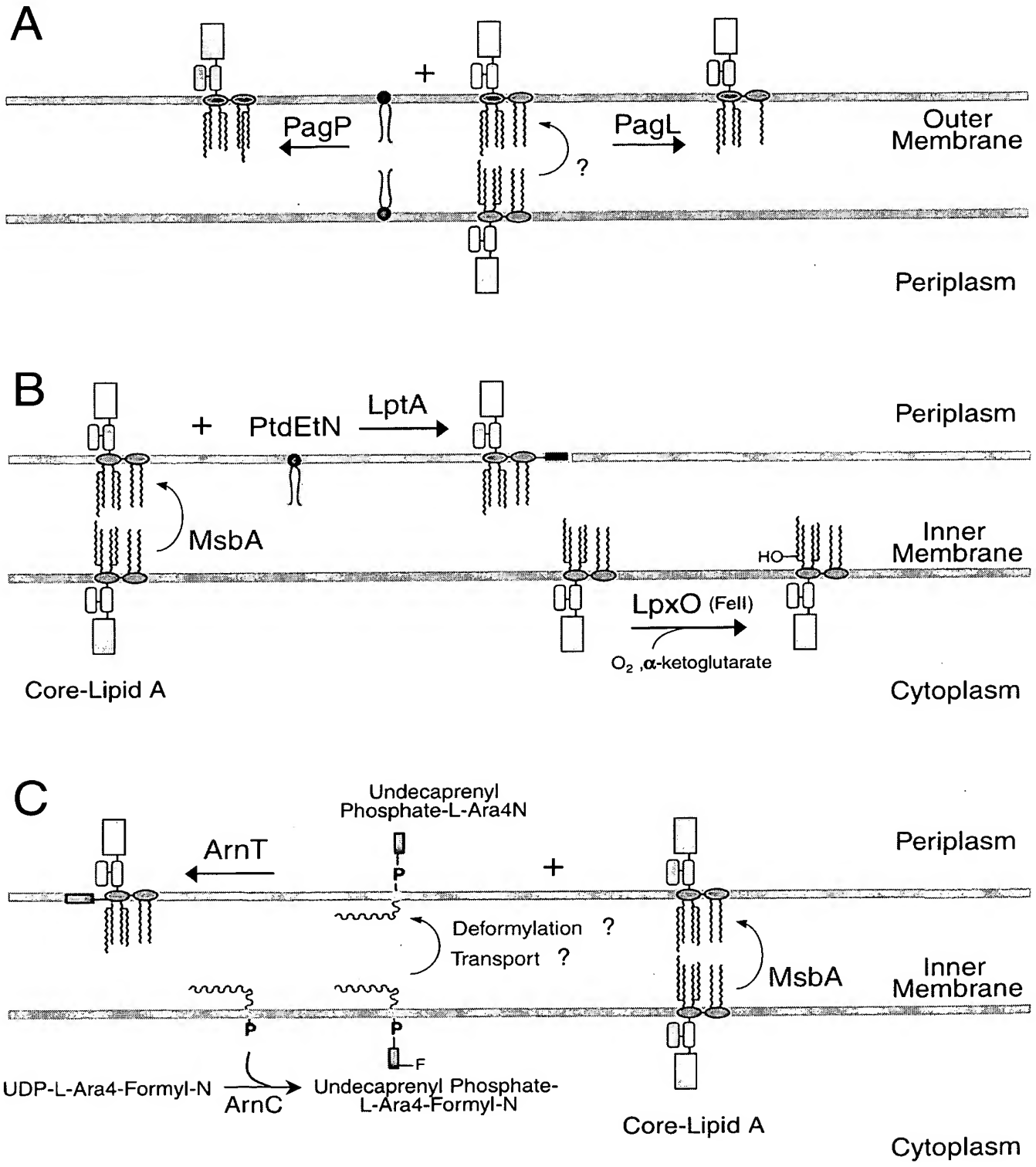
Much of the diversity seen in the lipid A structures from various Gram-negative bacteria is due to differences in their acylation patterns (Fig 3). When *E. coli* are cultured at 12 °C, a third acyltransferase termed LpxP adds a palmitoleoyl group (C16:1) in an acyloxyacyl linkage at the 2′ position, replacing LpxL activity (Carty et al. 1999). Incorporation of the unsaturated palmitoleoyl fatty acyl chain is thought to aid in the adjustment of outer membrane fluidity of the bacterium when shifted to lower temperatures. LpxP is 54% identical and 73% similar to *E. coli* LpxL and requires the Kdo dissacharide for enzymatic activity. How the cell determines the amount of laurate or plamitoleate to be incorporated into lipid A is still unclear, but approximately 80% of the lipid A residues of cells grown at 12 °C contain the unsaturated fatty acyl chain (Carty et al. 1999). Mutants lacking functional LpxP were not compromised for growth at 12 °C; however, they showed a 10-fold greater sensitivity to rifampicin and vancomycin, suggesting changes in the integrity of the outer membrane (Vorachek-Warren et al. 2002). A similar phenonmenon has been seen in strains of *Yersinia* where induction of palmitoleate is seen at 27 °C but not at 37 °C (Aussel et al. 2000; Bengoechea et al. 2003; Krasikova et al. 1999).

The lipid A of some pathogenic bacteria can be further modified by the addition of a palmitoyl group (C16), which results in a hepta-acylated lipid A structure (Guo et al. 1998) (Fig. 3). PagP, an outer membrane serine hydrolase (Fig. 4), transfers the palmitate residue from the sn-1 position of phospholipids to the lipid A anchor of LPS (Bishop et al. 2000; Hwang et al. 2002). It has been shown in *S. typhimurium* that PagP is under the control of the PhoP/PhoQ two-component regulatory system that is activated during Mg^{2+} limitation, a condition found within phagolysosomes. A low Mg^{2+} growth environment also promotes palmitoylation of the lipid A in *E. coli* and *Yersinia* (Guo et al. 1998) In *E. coli* and *S. typhimurium*, the fatty acyl chain is added to the 2 position on the proximal glucosamine of lipid A in an acyloxyacyl linkage (Zhou et al. 1999, 2000, 2001). A tBLASTn search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Altschul et al. 1990, 1997) reveals PagP homologues in *Shigella flexneri*, *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica*, *Legionella pneumophila*, *Yersinia pestis*, and the plant pathogen *Erwinia chrysanthemi*. PagP mutants of *S. typhimurium* and *L. pneumophila* both show increased sensitivity to certain CAMPs that are produced by the host organism during initial stages of infection (Guo et al. 1998; Robey et al. 2001). These structurally diverse antimicrobial polypeptides are important components of the host innate immune response, as they are found in macrophages, neutrophils, and at most mucosal surfaces (Tsubery et al. 2000; Vaara 1993). The addition of the palmitate group has been suggested to result in a more tightly packed LPS, thereby increasing the integrity of the outer membrane (Guo et al. 1997, 1998).

In the respiratory pathogen *B. bronchiseptica*, palmitate is added in a secondary acylation at the 3′-position of lipid A (Preston et al. 2003). Preston et al. (2003) showed that *B. bronchiseptica pagP* mutants are not compromised during the early stages of infection of the mouse respiratory tract but functional PagP is required for persistence of the organism. *Pseudomonas aeruginosa* lipid A also contains palmitate in an acyloxyacyl linkage at the 3′ position (Ernst et al. 1999) (Fig. 3). Interestingly, there is no PagP homologue in the *Pseudomonas* genome (Stover et al. 2000), but a PagP-like acyltransferase activity can be detected in membranes of this organism (M.S. Trent and C.R.H. Raetz, unpublished). The nuclear magnetic resonance solution structure of *E. coli* PagP has been determined and reveals an eight-stranded antiparallel β-barrel. The N and C termini face the periplasmic space and the proposed active site is found on the extracellular boundary of the outer membrane localized within high concentrations of the lipid A anchor of LPS (Hwang et al. 2002). In the future, it will be of interest to determine the molecular mechanisms by which PagP acquires its phospholipid acyl donor from the inner leaflet of the outer membrane. Comparison of the *Bordetella* homologue with *E. coli* PagP may reveal how the enzyme determines which side of the lipid A dissacharide to acylate and whether or not placement of the palmitoyl group affects resistance to key CAMPs.

In *S. typhimurium*, PhoP controls two additional enzymes, not found in *E. coli*, that modify its lipid A. PagL is a PhoP-activated lipase that removes the *R*-3-hydroxymyristate from the 3 position of lipid A (Trent et al. 2001*a*) (Fig. 3). Like PagP, PagL is a small outer membrane enzyme (~18 kDa) characterized by a type I signal peptide. The function of

Fig. 4. Schematic depiction of the topology of lipid A modifications found in *Escherichia coli* and *Salmonella*. PagL and LpxO are not present in *E. coli*.



PagL is unknown, given that *S. typhimurium* mutants lacking *pagL* display no obvious phenotypes (Trent et al. 2001). Since reduction of acyl chains lowers the endotoxic principal of lipid A (Loppnow et al. 1989; Rietschel et al. 1996), PagL-catalyzed deacylation of *Salmonella* lipid A might result in a lower or altered immunological response, possibly

aiding the bacterium in establishing a prolonged infection. The *pagL* gene is unique to strains of *Salmonella* but 3-*O*-deacylase activities have been reported in *P. aeurginosa* and in the symbiotic nitrogen-fixing bacteria *Rhizobium* spp. (Basu et al. 1999*a*). Investigations of lipid A from other pathogenic bacteria, such as *Helicobacter pylori* (Moran et al. 1997; Suda et al. 1997, 2001) (Fig. 3) and *Porphyromonas gingivalis* (Kumada et al. 1995; Ogawa 1993), reveal the absence of ester-linked fatty acyl chains at the 3-*O* and 3′-*O* positions, suggesting possible deacylases in these organisms.

Also absent in *E. coli* is the PhoP-activated *lpxO* gene. The lipid A of certain Gram-negative bacteria contains the secondary fatty acyl chain 2-hydroxymyristate rather than the myristate residue (Fig. 3). Heterologous expression of *lpxO* in *E. coli* is sufficient to induce O_2-dependent formation of 2-hydroxymyristate-modified lipid A, suggesting that *lpxO* is the structural gene for a novel dioxygenase. LpxO is thought to function as an aspartyl/asparaginyl β-hydroxylase that is required for the direct hydroxylation of the 3′ secondary myristoyl chain of *S. typhimurium* lipid A (Gibbons et al. 2000). However, no enzymatic assay has been developed for the LpxO-dependent hydroxylation of lipid A or its precursors. Homologues of LpxO can be found in several pathogenic bacteria, including *P. aeruginosa*, *B. pertussis*, *L. pneumophila*, and *Klebsiella pneumonia* (Altschul et al. 1990, 1997). The importance of this modification still remains unclear.

Modification and (or) removal of the lipid A phosphate groups

As is the case with the fatty acyl chains of lipid A, the 1- and 4′-phosphates attached to the dissacharide backbone can be modified and (or) removed. A number of Gram-negative bacteria contain latent enzymes capable of modifying the lipid A phosphates with 4-amino-4-deoxy-L-arabinose (L-Ara4N) and (or) phosphoethanolamine (pEtN) (Fig. 3). In *Salmonella*, the modification of the lipid A phosphates occurs upon activation of the transcriptional regulatory protein PmrA (Gunn et al. 1998). PmrA activation is induced by extracytoplasmic iron via the iron-sensing protein PmrB (Chamnongpol et al. 2002; Kato et al. 2003; Wosten et al. 2000). Additionally, PmrA is activated by the PhoP/PhoQ system during Mg^{2+} starvation (Gunn and Miller 1996; Kato et al. 2003; Kox et al. 2000). Finally, PmrA-activated genes can be induced by mild acid pH in a process that is independent of both PmrB and the PhoP/PhoQ system (Soncini and Groisman 1996).

Masking of the lipid A phosphates with the cationic sugar L-Ara4N reduces its net negative charge, thereby lowering its affinity for CAMPs. *Salmonella typhimurium* mutants unable to modify their lipid A with L-Ara4N are sensitive to CAMPs as well as polymyxin, a cationic acylated CAMP produced by Gram-positive bacteria (Gunn et al. 1998, 2000; Guo et al. 1997; Vaara 1992). Polymyxin binds to lipid A and kills Gram-negative bacteria in a manner that shares some common features with the cationic peptides of the innate immune system (Vaara 1992). Substitution of lipid A with L-Ara4N and phosphoethanolamine units is greatly elevated in polymyxin-resistant mutants of *S. typhimurium* (Helander et al. 1994; Vaara et al. 1981) and *E. coli* K-12 (Nummila et al. 1995). PmrA constitutive mutants of *S. typhimurium* with increased levels of phosphoethanolamine and L-Ara4N substituents survive longer inside neutrophils (Stinavage et al. 1989). Conversely, *pmrA*-deficient mutants of *S. typhimurium* show reduced virulence compared with wild-type strains when administered orally to BALB/c mice (Gunn et al. 2000).

The synthesis and attachment of the L-Ara4N moiety requires PmrA activation of at least seven genes at the *pmrE*(*ugd*) and *pmrHFIJKLM* loci (*pmrM* is not required) (Gunn et al. 1998, 2000). Based on bioinformatic analyses of the *pmr* loci, a pathway for the biosynthesis of UDP-L-Ara4N and transfer of the L-Ara4N unit to lipid A was proposed that is now supported by a significant amount of biochemical evidence (Baker et al. 1999; Zhou et al. 1999). The first gene to be characterized was the L-Aar4N transferase, ArnT (PmrK), that displays distant similarity to yeast protein mannosyltransferases. ArnT of *S. typhimurium* consists of 548 amino acid residues with 12 possible membrane-spanning regions (Trent et al. 2001*c*). The enzyme utilizes the novel carrier lipid undecaprenyl phosphate-α-L-Ara4N as its sugar donor, suggesting that the active site of the enzyme is located on the periplasmic side of the inner membrane (Trent et al. 2001*b*) (Fig. 4). In eubacteria, undecaprenyl-phosphate and undeacprenyl-diphosphate serve as lipid carriers for sugar residues that are transferred to acceptors located outside the cytoplasm, where sugar nucleotides are not available (Bugg and Brandish 1994; Koyama 1999). Although typically found linked to the 4′-phosphate, both phosphates of lipid A can be substituted with L-Ara4N in several organisms (Ernst et al. 1999; Guo et al. 1997; Zhou et al. 2001). However, enzymatic studies suggest that ArnT requires the Kdo sugars for modification of the 4′-phosphate group (Trent et al. 2001*c*).

The synthesis of UDP-L-Ara4N begins with the well-characterized enzyme Ugd (PmrE), which catalyzes the conversion of UDP-glucose to UDP-glucuronic acid (Baker et al. 1999; Gunn et al. 1998; Zhou et al. 1999). ArnA, previously known as PmrI, catalyzes oxidation of the sugar at the 4 position and its subsequent decarboxylation at the 6 position (Breazeale et al. 2002). This is followed by a transamination event at the 4 position yielding UDP-L-Ara4N that is catalyzed by ArnB (PrmH), a cytoplasmic protein containing a pyridoxal phosphate cofactor (Breazeale et al. 2003; Noland et al. 2002). Both ArnA and ArnB have been cloned, overexpressed, and their enzymatic products purified and thoroughly characterized (Breazeale et al. 2002, 2003). Interestingly, ArnA possesses a second catalytic domain having homology to methionyl-tRNA formyltransferase, which transfers the formyl group from *N*-10-formyltetrahydrofolate to the 4-amine of UDP-L-Ara4N (Breazeale et al. 2002).

The formylated sugar (UDP-L-Ara4-Formyl-N) is then transferred to an undecaprenyl-phosphate carrier lipid by the synthase ArnC (PmrF) (Fig. 4). Although no enzymatic data exist for ArnC, the protein has homology to the dolichoyl phosphate-mannose synthase of yeast (Baker et al. 1999; Gunn et al. 1998; Zhou et al. 1999). Like undecaprenol-phosphate carrier lipids, dolichol-phosphate serves as a carrier lipid for transport of sugars in higher organisms (Bugg and Brandish 1994; Burda and Aebi 1999; Orlean 1992; Staneloni and Leloir 1982). Following its transport across

the inner membrane, the formyl group must be removed by a deformylase to produce undecaprenyl phosphate-L-Ara4N, which, as described above, serves as the substrate for ArnT (Fig. 4) (Baker et al. 1999; Gunn et al. 1998; Zhou et al. 1999). Thus far, there are no candidates for the proposed transporter of the carrier lipid or the deformylase.

Unlike L-Ara4N addition, the importance of pEtN modification for CAMP resistance is still unclear. Recently, identification of a gene in *N. meningitidis*, designated *lpt*-3 (Mackinnon et al. 2002), has revealed a family of enzymes that play a role in pEtN modification of LPS. Lpt-3 is required for the addition of pEtN to the distal heptose sugar located in the inner core of *Neisseria* LPS (Mackinnon et al. 2002). A *Neisseria* homologue of *lpt*-3, named *lptA* (LPS pEtN transferase for lipid A), is required for pEtN modification of the phosphate groups of *N. meningitidis* lipid A (Cox et al. 2003) (Fig. 4). No enzymatic data have been presented for either Lpt-3 or LptA. However, a pEtN transferase activity that transfers pEtN from phosphatidylethanolamine to the phosphate groups of lipid A precursors has been identified in membranes of polymyxin-resistant mutants of *E. coli* (M.S. Trent, M. Reynolds, and C.R.H., Raetz, unpublished data).

Furthermore, Kanipes et al. (2001) identified a novel Ca^{2+}-induced enzyme that modifies the outer Kdo moiety of *E. coli* LPS with a pEtN group. This was the first report of a regulated LPS core modification. The Clusters of Orthologus Groups (COG) protein database (Tatusov et al. 1997, 2000, 2001) reveals six homologues of *lpt*-3 in *E. coli* alone (COG2194). Interestingly, the closest *E. coli* homologue to LptA is encoded by *yjdB* that is found at minute 93 of the *E. coli* genome downstream of the *pmrA/pmrB* locus. According to the COG database, the LPS pEtN transferase family is composed of 48 proteins within 20 different species. Generation of pEtN transferase mutants in various organisms will aid in the determination of the importance of pEtN modification to lipid A and to the core sugars during infection.

Masking the negatively charged phosphates of lipid A aids in resistance to antimicrobial peptides. However, some Gram-negative bacteria take an alternative approach by expressing enzymes that remove the lipid A phosphate groups from their LPS. During the biochemical synthesis of lipid A, LpxB catalyzes the condensation of one molecule of UDP-2,3-diacylglucosamine with one molecule of 2,3-diacylglucosamine 1-phosphate (lipid X) to form the disaccharide 1-phosphate precursor. The latter is then phosphorylated at the 4′ positon by LpxK to form lipid IV, a bisphosphorylated species (Fig. 2). Although the lipid A biosynthetic pathway is highly conserved, a number of Gram-negative bacteria, including *H. pylori* (Moran et al. 1997; Suda et al. 1997, 2001), *P. gingivalis* (Kumada et al. 1995), *Bacteroides fragilis* (Weintraub et al. 1989), *Francisella tularensis* (Vinogradov et al. 2002), and the symbiotic bacterium *Rhizobium leguminosarum* (Que et al. 2000*a*, 2000*b*), lack one or both phosphate groups from their lipid A anchors.

Recently, Karbarz et al. (2003) identified *lpxE*, the structural gene encoding a phosphatase that is selective for the 1-phosphate of *R. leguminosarum* lipid A. LpxE of *Rhizobium* is a 244 amino acid protein containing six predicted transmembrane domains. Based on the enzyme's predicted membrane topology, the proposed active site faces the periplasmic surface of the inner membrane. As reported by Karbarz et al. (2003), possible orthologs of LpxE are present in the bacterial pathogens *F. tularensis*, *Brucella melitensis*, and *L. pneumophila*. Recently, our laboratory has identified and characterized the 1-phosphatase of *H. pylori* (A.X. Tran and M.S. Trent, unpublished data). In *H. pylori*, removal of the 1-phosphate group appears to be followed by the addition of a phosphoethanolamine unit to form a glycosidic linkage (Fig. 3). This is in contrast with the phosphoethanolamine units of *E. coli* (Zhou et al. 1999), *S. typhimurium* (Zhou et al. 2001), and *N. meningitidis* (Kulshin et al. 1992), which are attached to the lipid A phosphate group to form a pyrophosphate linkage (Fig. 3). The enzymatic removal of the 4′-phosphate from lipid A and its precursors has also been detected in *R. leguminosarum* (Basu et al. 1999*b*; Brozek et al. 1996) and in *H. pylori* (A.X. Tran and M.S. Trent, unpublished data). Removal of the lipid A phosphate units may prove beneficial to pathogenic bacteria during infection by reducing the endotoxic properties of lipid A while at the same time reducing its affinity for antimicrobial peptides.

On the whole, the ability of various pathogenic bacteria to modify their LPS structure in response to environmental stimuli demonstrates the importance of LPS structure during infection. In general, the enzymes necessary for modification of lipid A are localized in the periplasmic space or the outer membrane (Fig. 4), possibly in order not to interrupt the conserved biosynthetic pathway. The characterization of the enzymes involved in LPS biosynthesis, its transport, and its modification will provide unique opportunities for the development of new antibacterial agents and vaccines. Modification of LPS represents one of the molecular mechanisms of microbial surface remodeling used by bacteria to help evade the innate immune response.

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